

Award Number: W81XWH-11-1-0367

TITLE: Analysis of Novel Prostate Cancer Biomarkers and their Predictive Utility  
in an Active Surveillance Protocol

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REPORT DATE: May 2014

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE May 2014		2. REPORT TYPE Annual Summary		3. DATES COVERED 1 May 2013 – 30 April 2014	
4. TITLE AND SUBTITLE  Analysis of Novel Prostate Cancer Biomarkers and their Predictive Utility in an Active Surveillance Protocol				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-11-1-0367	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Adam S. Feldman, M.D., M.P.H.  E-Mail: afeldman@partners.org				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)  Massachusetts General Hospital Boston, MA 02114				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT The Research Project supported by this DOD PCRP Physician Research Training Award investigates novel biomarkers for prostate cancer and investigation of Active Surveillance of low risk prostate cancer. In the third year of this award I have continued to investigate potential prostate cancer markers using Western Blot, ELISA, and RCC. Proteins that I have investigated include Semenogelin-2, Lactoylglutathione Lyase, Hepsin, Leukocyte Elastase Inhibitor (SERPINB1), Alpha-1-Antichymotrypsin (Serpina3), Growth-Inhibiting Protein 12 (GIP 12), Prohibitin, Radixin, Taldo1, Fructose-Bisphosphate Aldolase A, Lactate Dehydrogenase A, CD63, Cytochrome C, Ras-related protein RAB-3A, Macrophage Capping Protein, 10kd Heat Shock Protein, Annexin A3, Sorbitol Dehydrogenase, Fibrinogen Beta Chain Precursor, Creatine Kinase B-Type, Annexin A1, Cystatin B, and AZI. We have continued to have promising results in TIMP-1 and now also Seminogelin-2. In addition to our laboratory work, we have built on our previous work in further developing our clinical database and evaluating our cohort of men on active surveillance (AS) for low risk prostate cancer. Our database now consists of over 600 men on active surveillance and we recently submitted our manuscript for publication describing our initial cohort of 469 men.					
15. SUBJECT TERMS: none listed					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
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**Introduction:**

The Research Project supported by this DOD PCRP Physician Research Training Award investigates novel biomarkers for prostate cancer detection and prediction of disease outcome. The goals and objectives of this study are summarized by the Specific Aims: 1. Evaluate the relative levels of expression of our panel of candidate protein biomarkers in urine, tissue and serum from patients with prostate cancer compared with normal controls to identify prostate cancer specific biomarkers. 2. Evaluate the relative urine, tissue and serum levels of these prostate cancer specific biomarkers within our entire active surveillance (AS) cohort to identify accurate biomarkers predictive of indolent vs. progressive prostate cancer. The funding from this Physician Research Training Award provides salary support for Dr. Adam S. Feldman to secure protected time as a translational and clinical investigator in prostate cancer research. It also provides salary support for a Research Assistant for this project.

**Body:**

The first year of my DOD PCRP PRTA was very productive from both a translational laboratory and clinical research standpoint. In summation, I used mass spectrometry (MS) to quantitatively compare the entire urinary proteome and identify differentially expressed proteins in the urine from men with prostate cancer as compared with those found in controls. The MS analysis identified >1400 unique proteins, comparative analysis revealed 55 potential prostate cancer specific proteins, and using bioinformatic database analyses, we narrowed this list to 20 biologically relevant proteins. Using semi-quantitative Western blot, we investigated several proteins on the list of 20 relevant proteins including Leukocyte Elastase Inhibitor, Annexin A1, Plastin-2, Vimentin, and Tissue Inhibitor of Matrix Metalloproteinase Type 1 (TIMP-1). We used urine specimens of 56 men, both from PrCA and Controls. These urine specimens were selected from our urine biospecimen bank, prospectively obtained and developed from our urologic oncology clinic at Massachusetts General Hospital. In TIMP-1, we found a significant difference in TIMP-1 expression between men with Gleason 3+3 disease and men with Gleason 7 or greater.

In the second year of my DOD PCRP PRTA, I further explored the compelling data from the TIMP-1 Western blots and returned to my original list of 55 differentially expressed potential prostate cancer specific proteins to assess other possible candidate markers. Looking at TIMP-1, we used Enzyme-Linked Immunosorbent Assays (ELISA) and Immunohistochemistry (IHC) to corroborate the data we found in Western blot analyses. Using IHC, we were able to show increased staining for Gleason 8 or higher, compared to lower Gleason scores supporting our previous Western blot data and further pointing to the potential of TIMP-1 as a relevant biomarker for prostate cancer. For ELISA analysis of TIMP-1 expression we used the same cohort of 56 men, both PrCA and Controls, as analyzed by Western blot. Although we demonstrated differential expression across our cohort, we were unable to effectively reproduce the results we found in Western blot. This discrepancy between Western blot and ELISA results were consistent across two separate commercially available ELISA kits (R&D Systems, Minneapolis, MN and EMD

Millipore, Billerica, MA), suggesting that possibly the Western blot antibodies and ELISA antibodies were measuring separate epitopes and therefore demonstrating different results. In my second year we also returned to my original list of 55 differentially expressed potential prostate cancer specific proteins, using both Western blot and ELISA analyses with several representative specimens as an initial evaluation to screen for those proteins with potential clinical relevance.

In this past third year of my DOD PCRP PRTA, I pursued the promising Western blot candidates from year two, Semenogelin-2, Lactoylglutathione Lyase, Hepsin, Leukocyte Elastase Inhibitor (SERPINB1), Alpha-1-Antichymotrypsin (SerpA3), and Growth-Inhibiting Protein 12 (GIP 12). After the promising preliminary test Western blot for each of the proteins, I tested a larger cohort of patients (56 total of PrCA and Normal Controls). The initial trend of potentially significant Westerns did not hold true with the larger cohort (Figures 1 and 2). We inevitably found the Normal Controls to have high enough levels of our potential biomarkers to render the results insignificant. One protein that continues to show promise is Semenogelin II, but the proper antibody and optimized Western blot protocol has required significant troubleshooting and is requiring continued investigation.

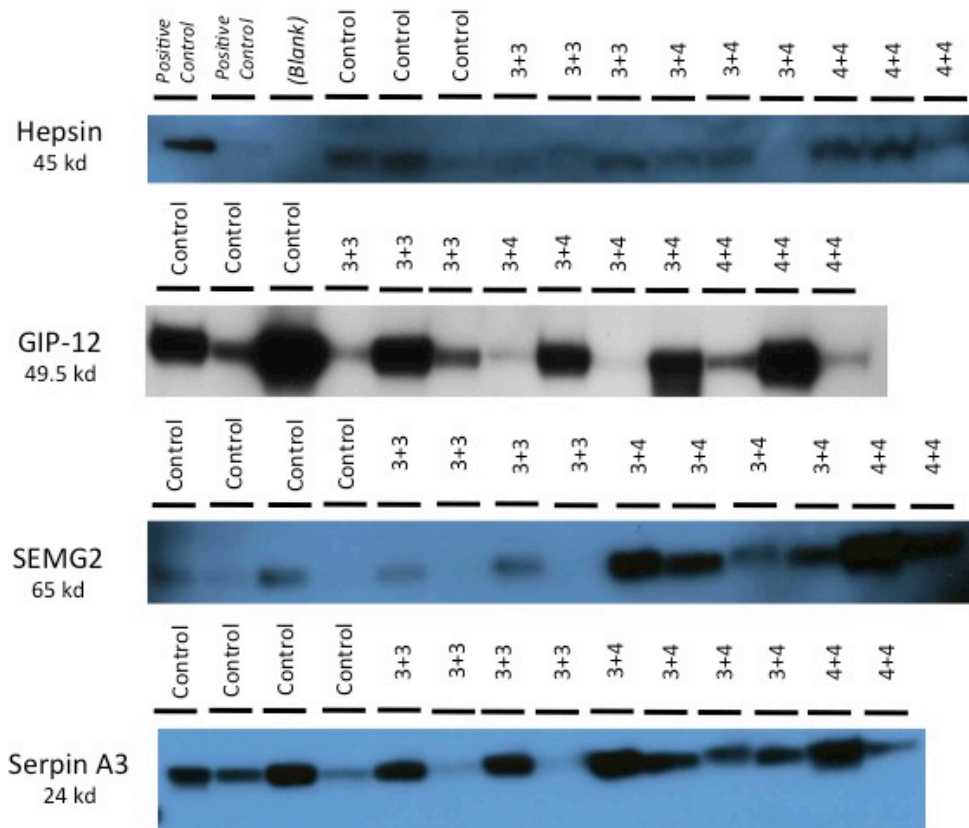


Figure 1: Representative Western blot analysis of urinary expression of Hepsin, Growth-Inhibiting Protein 12 (GIP 12), Semenogelin-2 (SEMG2), and Alpha-1-Antichymotrypsin (SerpinA3).

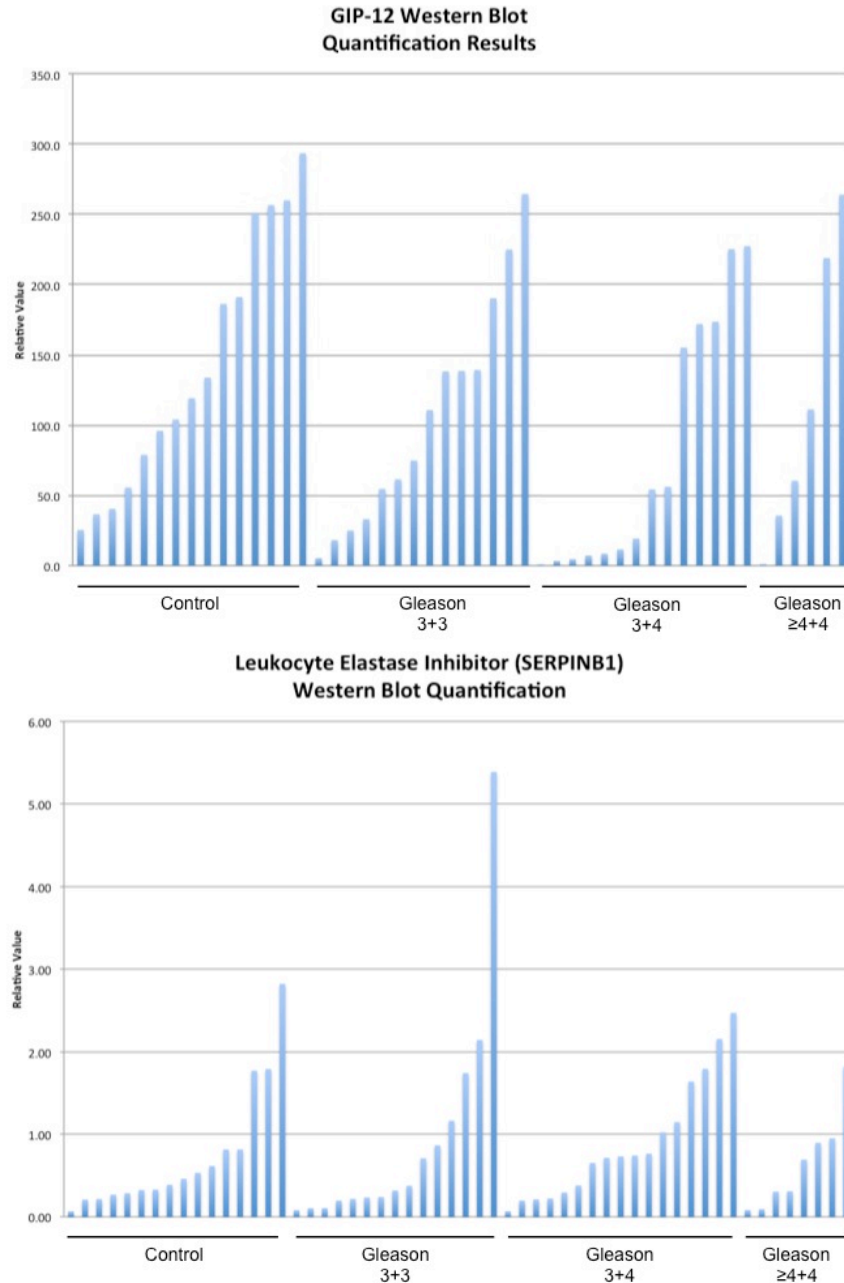


Figure 2: Western blot analysis of Growth-Inhibiting Protein 12 (GIP 12) and Leukocyte Elastase Inhibitor (SERPINB1)

Returning to the list of 55 differentially expressed potential prostate cancer specific proteins, we further investigated our initial findings for a number of candidates. As a first pass evaluation of these proteins, we used a single Western blot with several representative specimens as an initial evaluation to screen for those proteins with potential clinical relevance. In 2012, we looked the protein candidates: Prohibitin, Radixin, Taldo1, Fructose-Bisphosphate Aldolase A, Lactate Dehydrogenase A, CD63, Cytochrome C, Ras-related protein RAB-3A, Macrophage Capping Protein, 10kd Heat Shock Protein, Annexin A3, Sorbitol Dehydrogenase, Fibrinogen Beta Chain Precursor, and Creatine Kinase B-Type. After testing these candidates with follow up Western blots

with a larger cohort and finely tuned individual protocols, we confirmed our initial finding of insignificant results (Figure 3). We also tested other biologically relevant candidates Annexin A1, Cystatin B, and AZI, unfortunately with similarly insignificant results.

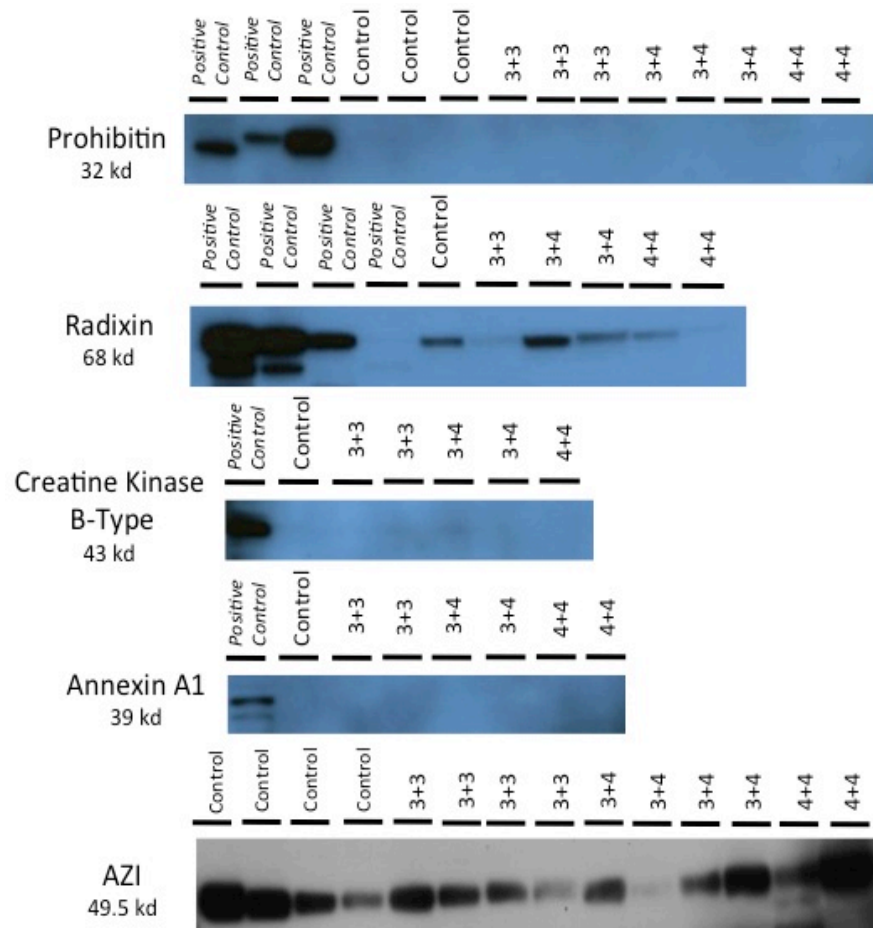


Figure 3: Representative Western blot analysis of urinary expression of Prohibitin, Radixin, Creatine Kinase B-Type, Annexin A1, and AZI.

A potential issue with our Western blot protocol was the amount of protein per patient sample that we were loading into the Western blot gels. Initially we wanted to ensure we could detect our protein candidates that may be present in the patient samples in minute amounts by loading 75ug of protein per patient into the Western blot gel. There was a possibility that our results were being skewed by the large amount of protein we were loading, so we corrected for that by changing the protocol to 40ug of protein. After comparing the two different Western protocols for our most promising candidates, including TIMP-1, we found that there was no marked difference in the results (Figure 4). This validates our prior TIMP-1 Western blot data, and reinforces our continued pursuit for a panel of biomarkers to be used in both diagnosis and prognosis discovery.

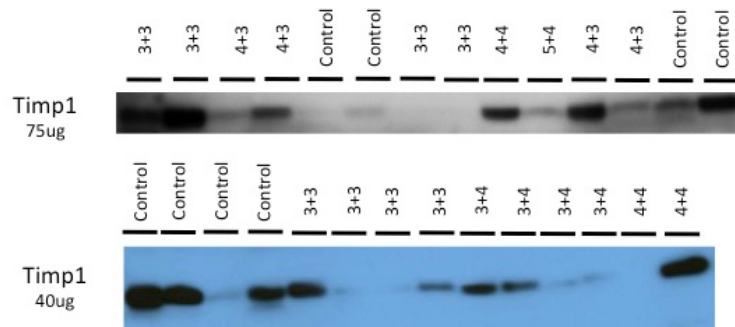


Figure 4: Representative Western blot analysis of urinary expression of 75 ug of protein and 40 ug of protein for Tissue Inhibitor of Matrix Metalloproteinase Type 1 (TIMP-1).

To further validate our results from Western blot analysis, we pursued Enzyme-linked Immunosorbent Assay (ELISA) analysis of biologically relevant and promising candidates Prohibitin, 10kd Heat Shock Protein, and Growth-Inhibiting Protein 12 (GIP 12). We used ELISA kits from *Uscn Life Science Inc.* (E90442Hu, E91501Hu, and E90780Hu respectively). After using multiple protocols and kits, we found the Western results to be similarly difficult to replicate as they were for Timp-1 in 2012 (Figure 5). To restate our hypothesis: this discrepancy could be caused by a few factors. First, the Western blot procedure requires denaturing of the proteins in the urine samples and ELISA does not. We addressed this issue by using Dithiothreitol (DTT) to denature the proteins in the urine samples for ELISA, but found that the addition of DTT inhibited the efficacy of the ELISA. A second potential issue is the diverse composition of urine itself and the differences in sample preparation required for Western blot compared with that for ELISA. Unlike the Western blot, the ELISA protocol detects protein within a sample of isolated protein. Western blots, however, depend on polyacrylamide gel electrophoresis followed by electromotive transfer of the protein from the gel onto a polyvinylidene difluoride (PVDF) membrane. This process is essentially a built in purification process during Western blot preparation and is not present in ELISA preparation. Therefore, this may affect the detectable presence of low molecular weight and low abundance proteins in a sample and therefore lead to discrepancies in apparent expression as measured by these two methods. We attempted to correct for this issue when processing the urine samples by diluting, and then concentrating to filter out some of the solutes. However, it may be that we cannot successfully purify or prepare the urine samples in an effective manner to replicate the Western blot results.

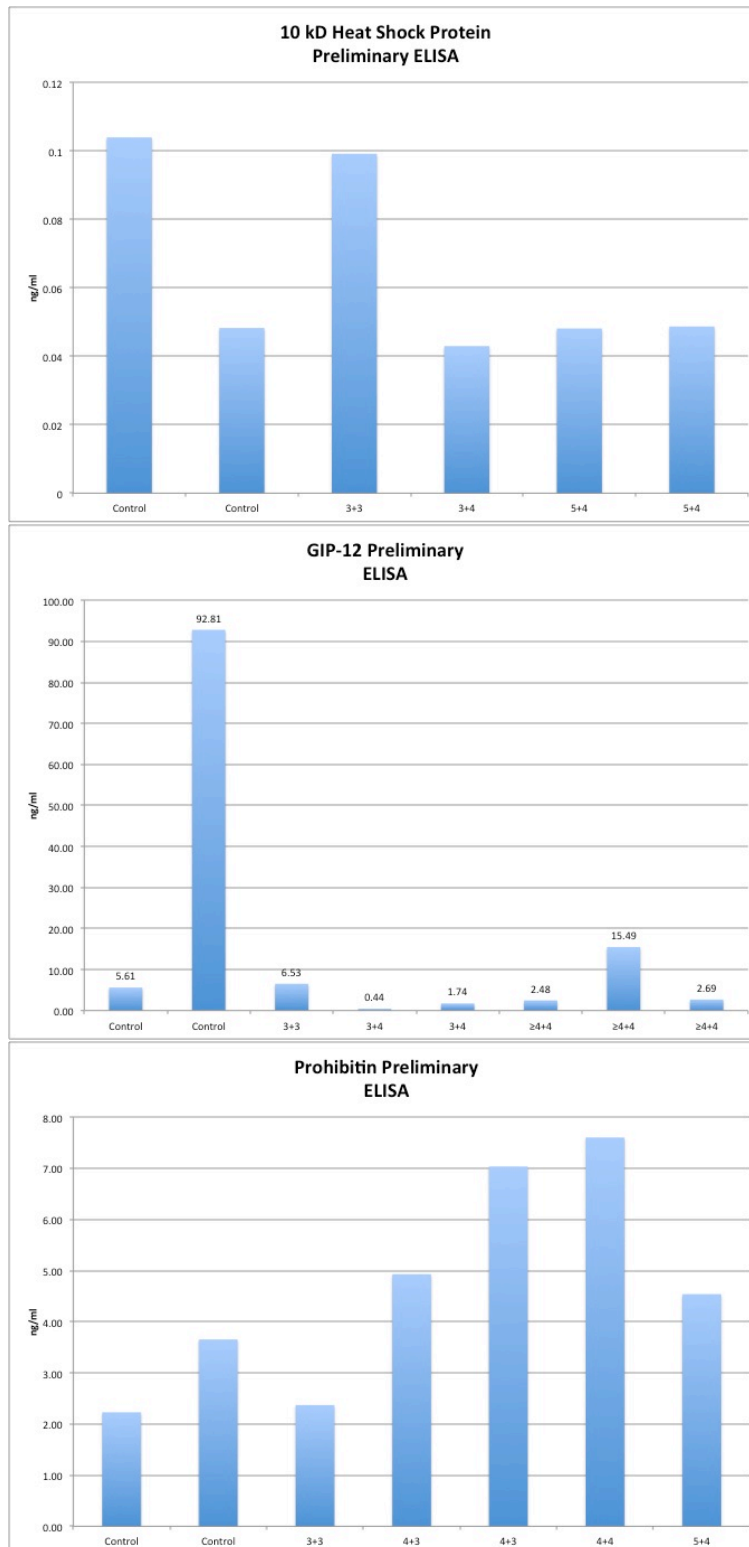


Figure 5: Enzyme Linked Immunosorbant Assay analysis of 10kd Heat Shock Protein, Growth-Inhibiting Protein 12 (GIP 12), and Prohibitin.

The third method of validation we used was Immunohistochemistry (IHC) staining of relevant tissues. Our previous promising results from 2012 with Tissue Inhibitor of Matrix Metalloproteinase Type 1 (TIMP-1), showed a marked difference between staining in Normal Control patients and PrCA patients. For the next validation step, we used a commercially available tissue micro array (US Biomax Inc.) to expand the sample of both Normal Control and PrCA patients being examined (Figure 5). Unfortunately, there were no significant results from various IHC's with optimized protocols for several antibodies. Upon examination of the commercially available tissue micro array by Pathologists Dr. Chin-Lee Wu and Dr. Liangzhe Wang, it was found that the tissue samples on the commercially obtained microarray were not Gleason graded or scored reliably well. One of the samples on the slide was not even prostate tissue and was denoted as such. To correct for unreliable tissue, I next used tissue samples from Dr. Wu's patient library. We are in an ongoing process of optimizing and validating a TIMP-1 IHC antibody protocol to corroborate our results from 2012.

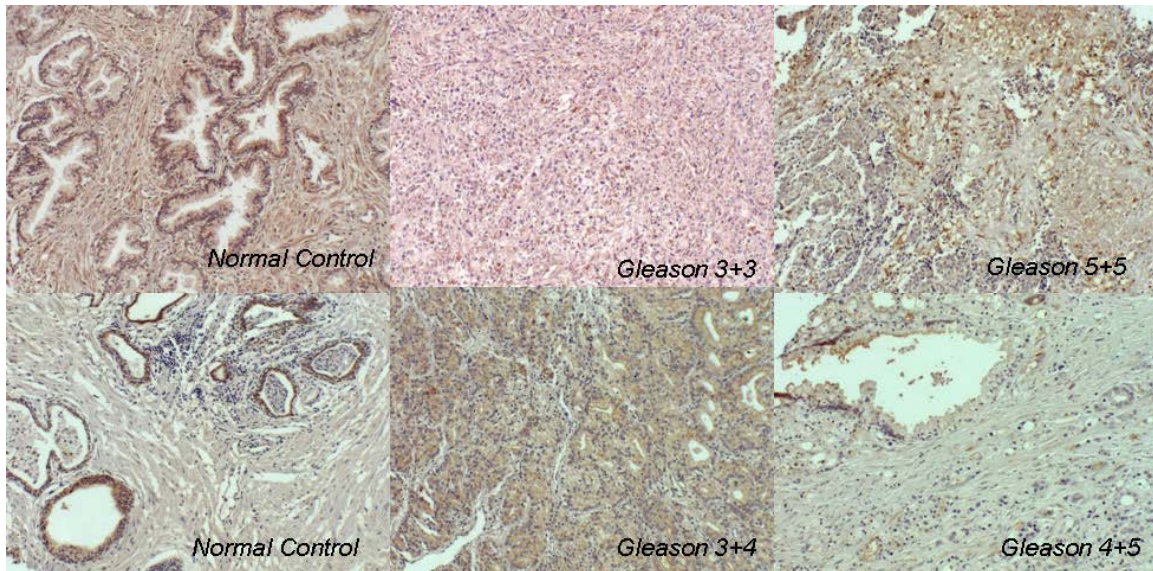


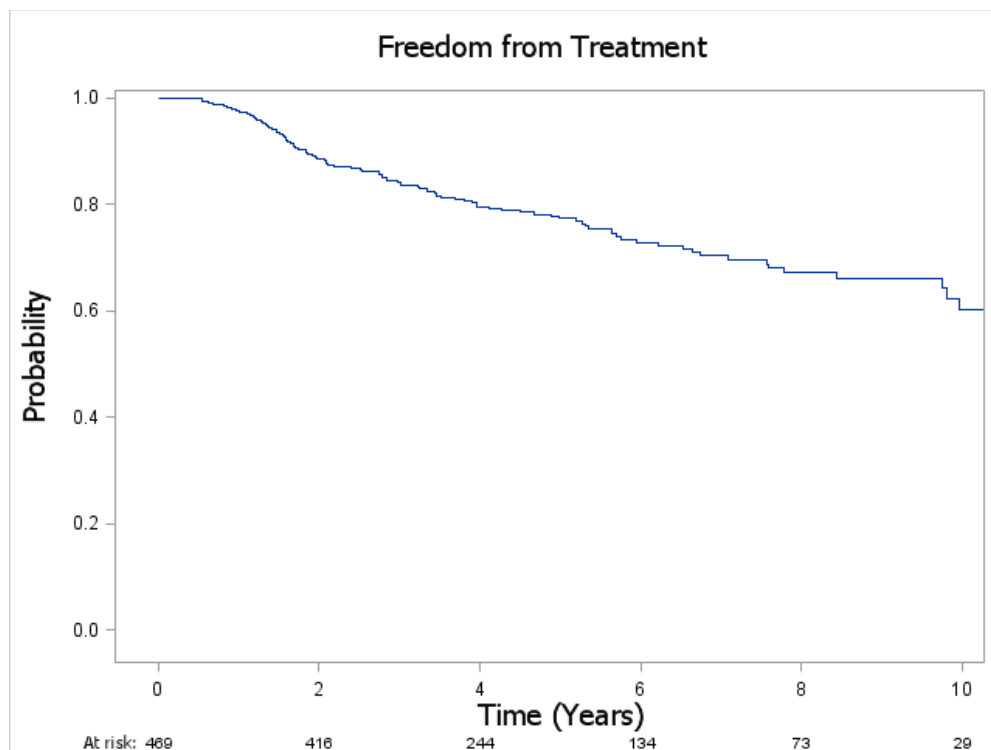
Figure 6: Representative IHC staining for TIMP-1 in prostate cancer tissue.

In addition to our laboratory work, we have built on our previous work in further developing our clinical database and evaluating our cohort of men on active surveillance (AS) for low risk prostate cancer. Our database now consists of over 600 men on active surveillance for low risk prostate cancer identified through billing and pathology records. We are continuing to update this database and investigate clinical outcomes in this cohort. Although AS had been practiced throughout this entire period, in 2008 our group agreed upon formal guidelines for AS at our institution. Inclusion criteria included Gleason  $\leq 6$ , Gleason 7 in select patients with low volume, no more than 3/12 cores

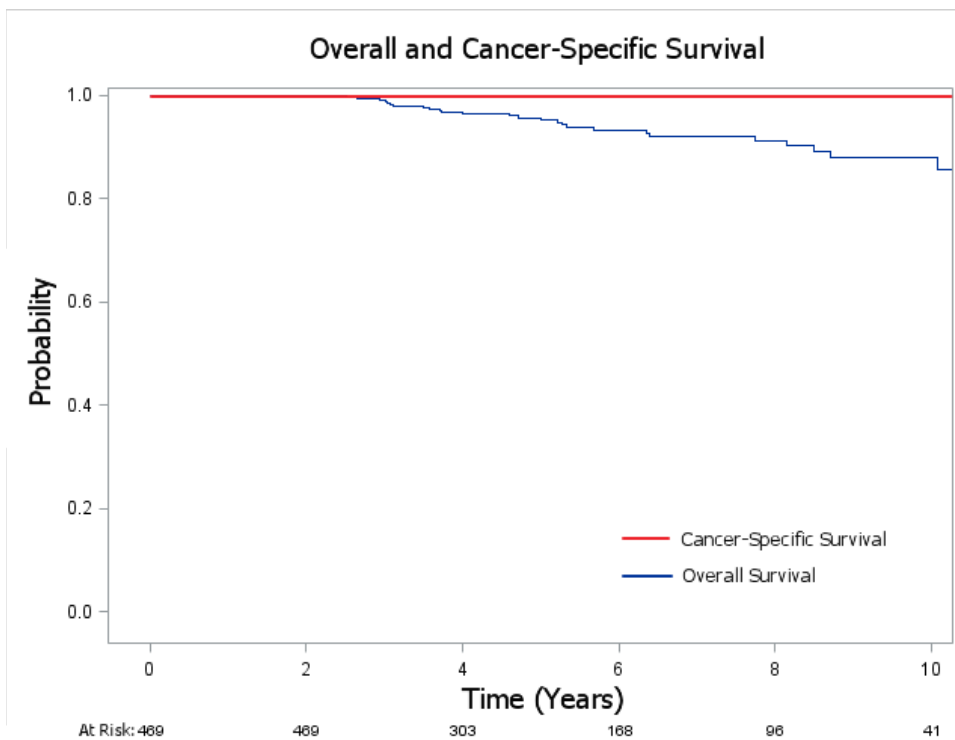
positive with  $\leq 20\%$  in each core, and PSA  $< 10$ . Our AS follow-up protocol involves PSA testing and a digital rectal examination every four months for one year, followed by every six months for two years, and then annually. Those on AS also have a mandatory repeat 12-core biopsy 12-18 months after initial diagnosis. Additional biopsies are at the discretion of the treating physician.

We have presented data from our initial cohort of 469 men at national meetings and recently submitted our manuscript for publication. In our report, 469 men with a median age at diagnosis of 68.1 (range, 38.8-82.7 years) were followed for a median of 4.8 years (range, 2-14.5 years). The median PSA at diagnosis was 5.1 ng/mL (range, 0.4-19.2) with 94% having a PSA  $< 10$  ng/mL. Overall, 98.3% (461/469) of patients were Gleason 6, 1.7% (8/469) were Gleason 3+4=7, and 94.0% (441/469) were stage T1c. Freedom from intervention was 77% at 5 years and 62% at 10 years (Figure 7). A total of 116 (24.7%) patients received treatment during the course of surveillance. Reasons for intervention included; 44.8% (52/116) for pathologic progression, 30.2% (35/116) for PSA progression, 12.1% (14/116) due to patient preference, 5.2% (6/116) for DRE progression and 4.3% (5/116) for metastatic disease. Of patients who were treated, 56 (48.3%) received radiation, 26 (22.4%) received surgery, 17 (14.7%) received brachytherapy and 17 (14.7%) received androgen deprivation therapy. On pathologic review after radical prostatectomy, 4/26 (15.4%) patients were pathologic stage T3. Cancer-specific survival was 100% at 5 and 10 years and overall survival was 95% at 5 years and 88% at 10 years (Figure 8).

**Figure 7:** Kaplan Meier curve showing freedom from intervention.

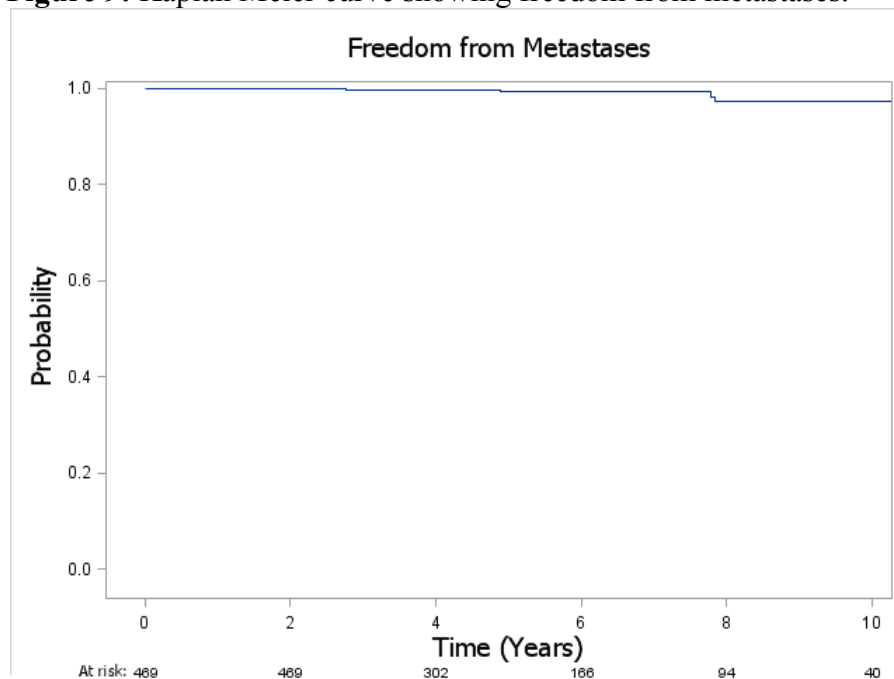


**Figure 8:** Kaplan Meier curve showing cancer specific and overall survival.

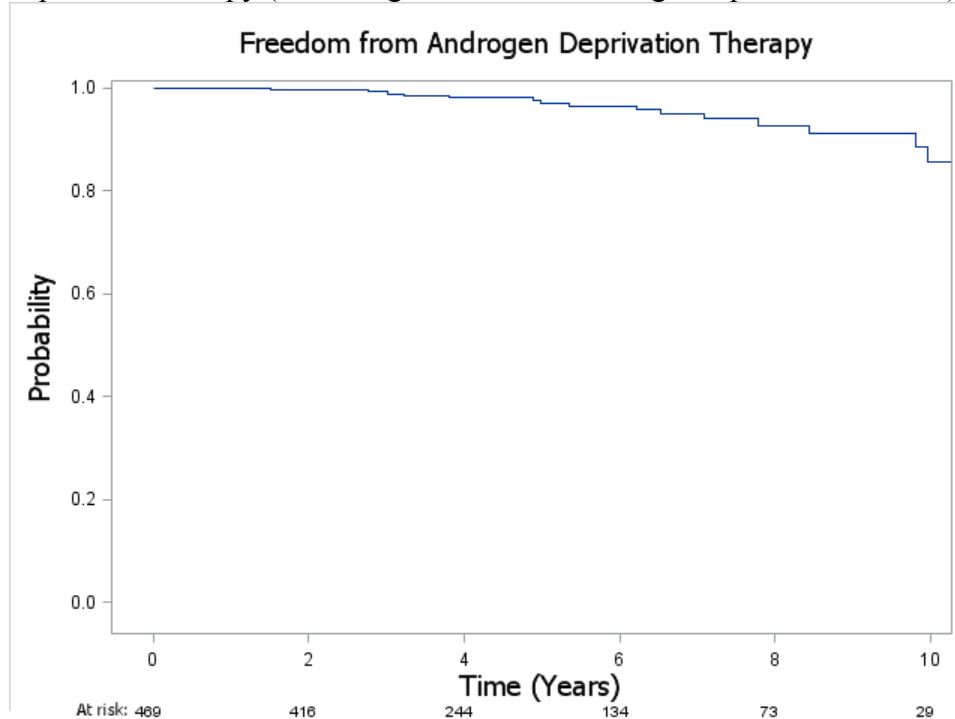


Metastases-free survival was 99.3% at 5 years and 97.3% at 10 years (Figure 9). Freedom from androgen deprivation was 97.2% at 5 years and 85.8% at 10 years (Figure 10).

**Figure 9:** Kaplan Meier curve showing freedom from metastases.



**Figure 10:** Kaplan Meier curve showing freedom from primary or salvage androgen deprivation therapy (excluding short-course ADT given prior to radiation).



In addition to significant research accomplishments, I continue to meet my goals within the training program of this grant. I meet regularly with my two mentors, Drs. Matthew Smith and Bruce Zetter. In our regular meetings, we not only discuss research progress, but also focus on career planning and guidance. I attend regular urologic oncology clinical and research conferences at our institution and both attend and present at regional and national scientific meetings. I attend regular laboratory research meetings both for our own research progress, as well as reviewing other associated research in the current literature. I also have participated multiple times as an invited reviewer of research grant applications for the Prostate Cancer Foundation Young Investigator Awards and Challenge Awards.

#### **Key Research Accomplishments:**

- Identification of panel of biologically relevant proteins in urine which may be prostate cancer specific.
- Validation of our preliminary Western blot results showing TIMP-1 to be more highly expressed in the urine of men with intermediate or high risk disease, in Western blot and possibly Immunohistochemistry staining as well.
- Continued analysis of a large database of our cohort of men with low risk prostate cancer on active surveillance.

## Reportable Outcomes:

- \*Preston MA, \***Feldman AS**, Coen JJ, McDougal WS, Smith MR, Paly JJ, Carrasquillo R, Wu CL, Dahl DM, Barrisford GW, Blute MB, Zietman AI. Active surveillance for prostate cancer: need for intervention and survival. *Submitted for publication*. \*Co-first Authorship
- Gershman B, Zietman AL, **Feldman AS**, McDougal WS. Transperineal Template-Guided Prostate Biopsy for Patients with Persistently Elevated PSA and Multiple Prior Negative Biopsies. *Urol Oncol*. 2013 Oct;31(7):1093-7.
- Pollock CB, McDonough S, Wang VS, Lee H, Ringer L, Li X, Prandi C, Lee RJ, **Feldman AS**, Koltai H, Kapulnik Y, Rodriguez OC, Schlegel R, Albanese C, Yarden RI. Strigolactone analogues induce apoptosis through activation of p38 and the stress response pathway in cancer cell lines and in conditionally reprogrammed primary prostate cancer cells. *Oncotarget*. 2014 Apr 2. [Epub ahead of print]
- **Feldman AS**, Wu CL, Fergus M, Lee RJ, Schlegel R, Boehm J, Garraway L, Zetter BR, Smith MR, Albanese C. Development of conditionally reprogrammed cells in culture from human prostate cancer specimens. Presented at the Prostate Cancer Foundation Scientific Retreat. 2013.
- Preston MA, Batista J, Carlsson S, Gerke T, Dahl D, **Feldman AS**, Gann PH, Vickers A, Stampfer MJ, Mucci LA. Prostate-specific antigen (PSA) levels in men <60 years of age predicts lethal prostate cancer. Abstract presented at the American Urological Association national meeting, May 2014.

## Conclusion:

In summary, these past three years of my DOD PCRP PRTA have been very productive. We validated our study of novel TIMP-1 protein in Western blot analysis and continue to pursue Immunohistochemistry analyses of cancer tissue. We are continuing to investigate our list of biologically relevant candidate prostate cancer biomarkers and have once again demonstrated promising results with other candidates.

In addition to success in our laboratory work, we have also made significant accomplishments in continued analysis of our large cohort of men on active surveillance for prostate cancer and continue to build this database and further assess multiple important clinical questions.

This work is very relevant to current clinical practice in prostate cancer and meets any potential “so what” criteria. New diagnostic and predictive biomarkers with improved performance characteristics than prostate specific antigen (PSA) are sorely needed. The work funded by this grant directly addresses that challenge and we are already beginning to produce results toward that goal. In addition, it is clear that we have historically over-treated low risk prostate cancer. Active surveillance is a management strategy for low risk disease which will help ameliorate the problem of overtreatment. Our large database of men on active surveillance will help us to understand the safety, efficacy and outcomes of this strategy and will help us better select men for AS in the future. Biomarker analysis

within this cohort will also help us better understand who truly has very low risk disease and can safely avoid radical treatment.